

An Orally Available Small-Molecule Inhibitor of c-Met, PF-2341066, Reduces Tumor Burden and Metastasis in a Preclinical Model of Ovarian Cancer Metastasis¹

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Abstract

Deregulated expression of the hepatocyte growth factor (HGF) receptor, c-Met, in cancer contributes to tumor progression and metastasis. The objective of this study was to determine whether blocking c-Met with an orally available c-Met inhibitor, PF-2341066, reduces tumor burden and increases survival in a xenograft model of ovarian cancer metastasis. Treatment of mice injected interperitoneally with SKOV3ip1 cells showed reduced overall tumor burden. Tumor weight and the number of metastases were reduced by 55% ($P < .0005$) and 62% ($P < .0001$), respectively. Treatment also increased median survival from 45 to 62 days ($P = .0003$). *In vitro*, PF-2341066 reduced HGF-stimulated phosphorylation of c-Met in the tyrosine kinase domain as well as phosphorylation of the downstream signaling effectors, Akt and Erk. It was apparent that inhibition of the pathways was functionally important because HGF-induced branching morphogenesis was also inhibited. In addition, proliferation and adhesion to various extracellular matrices were inhibited by treatment with PF-2341066, and the activity of matrix metalloproteinases was decreased in tumor tissue from treated mice compared with those receiving vehicle. Overall, these data indicate that PF-2341066 effectively reduces tumor burden in an *in vivo* model of ovarian cancer metastasis and may be a good therapeutic candidate in the treatment of patients with ovarian cancer.

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Introduction

Ovarian cancer has the highest mortality rate of any gynecologic malignancy and is the fifth leading cause of cancer death in women [1]. Current treatment protocols for patients with advanced stage disease involve surgical debulking followed by six cycles of intravenous or intraperitoneal platinum-based chemotherapy [2]. Despite this aggressive treatment, the average time of clinical remission is just 2.5 years [3] and approximately 20% of women never achieve remission [2]. These statistics clearly underscore the need to identify new molecular targets that can be translated to the clinical treatment of women with ovarian cancer.

c-Met is a receptor tyrosine kinase with one high-affinity ligand, hepatocyte growth factor/scatter factor (HGF/SF). On binding of HGF, c-Met undergoes dimerization and tyrosine residues in the cytoplasmic domain are transphosphorylated, generating docking sites for SH2-containing adapter proteins including Grb2, Shc, c-Cbl, and Gab1. The adaptor proteins then recruit intracellular signaling transduc-

ers that activate, among others, *ras*-mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and the signal transducers and activators of transcription signaling pathways [4,5]. Activation of the pathways results in a plethora of biologic responses such as proliferation, cell cycle progression, migration, angiogenesis, and invasion, all of which contribute to the tumorigenicity of cancer cells.

Abbreviations: ALK, anaplastic lymphoma kinase; ECM, extracellular matrix; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase

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In normal physiology, the expression of c-Met is constrained to epithelial cells, whereas the expression and secretion of HGF is restricted to stromal cells. Overexpression of the receptor and its ligand has been implicated in a number of different cancers, including those of the breast, gastric system, and lung [4]. We have previously shown that c-Met is overexpressed in ovarian cancer and that its targeting by small interfering RNA (siRNA) inhibited adhesion, invasion, peritoneal dissemination, and tumor growth *in vivo* by blocking the fibronectin receptor $\alpha_5\beta_1$ [6]. In addition, we and others have shown a relationship between advanced stage disease, poor prognosis, and increased expression of c-Met in ovarian cancer [6–9]. Approximately 40% to 60% of all tumors from patients with ovarian cancer overexpress c-Met [8–11]. These data suggest that c-Met could be an important therapeutic target in the treatment of ovarian cancer, justifying the exploration of anti-c-Met treatment strategies for this disease.

Several strategies aimed at inhibiting both c-Met and HGF are currently being pursued. HGF variants, c-Met decoy receptors, neutralizing antibodies and small-molecule inhibitors have proven to be effective in inhibiting c-Met signaling in preclinical models of certain human tumors [12,13]. For example, a monoclonal antibody against c-Met, MetMab, functions by blocking HGF from binding to the receptor and the subsequent activation of the signaling pathway. MetMab was found to have potent antitumor activity and to increase survival in an orthotopic model of pancreatic cancer [14]. Whereas many small-molecule inhibitors of c-Met have been identified [15–17], these inhibitors often fail to reach the clinic because of poor bioavailability.

Given the many preclinical studies that indicate that the inhibition of c-Met is a viable therapeutic strategy, and the consistent finding that c-Met overexpression has prognostic value in ovarian cancer, we sought to determine the efficacy of an orally available inhibitor (PF-2341066) against c-Met in ovarian cancer. PF-2341066 is a novel small-molecule inhibitor highly specific against c-Met and anaplastic lymphoma kinase (ALK). Zou et al. showed that it is a potent ATP-competitive inhibitor of human c-Met kinase with a mean K_i of 4 nM [18,19]. Evaluation of the inhibitor against more than 120 different kinases revealed that PF-2341066 is more than 100 times more selective toward c-Met than the majority (>90%) of kinases tested. In addition, the inhibitor displayed potent activity against mutant c-Met variants that had been previously found to contribute to oncogenesis [20].

In the present study, we demonstrate that PF-2341066 reduces tumor burden and increases survival in a mouse model of ovarian cancer. The antitumor activity of PF-2341066 is mediated, at least in part, by reduced proliferation, adhesion, invasion, and induction of apoptosis.

Materials and Methods

Reagents and Cell Lines

PF-2341066 was provided by Pfizer Global Research and Development (La Jolla, CA) [19]. The extracellular matrices (ECMs), collagen type 1, fibronectin, and vitronectin and the antibody against total FAK were purchased from BD Biosciences (Bedford, MA). Anti-phospho-c-Met (Tyr^{1230/1234/1235} and Tyr¹⁰⁰³) and phospho-FAK (Tyr⁸⁶¹) polyclonal antibodies for immunoblot analysis were obtained from BioSource International (Camarillo, CA). Total c-Met (C-28) and normal mouse immunoglobulin G were purchased from

Santa Cruz Biotechnology (Santa Cruz, CA). Total Src and phospho-Src were purchased from Millipore (Billerica, MA). Antibodies against phospho-Gab1, Gab-1, phospho-c-Met (Tyr¹³⁴⁹) p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), Akt, phospho-Akt (Ser473), β -actin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling (Beverly, MA). The human ovarian cancer cell line CaOV3 was purchased from the American Type Culture Collection (Rockville, MD). SKOV3ip1 and HeyA8 cells were from Dr. Gordon B. Mills (MD Anderson Cancer Center, Houston, TX).

Xenograft Model

SKOV3ip1 cells (1×10^6) were injected i.p. into 5- to 7-week-old female athymic nude mice (NCI-Fredrick, MD) [21]. For the studies with a defined end point, tumors were allowed to grow for 8 days after injection and then treatment with 50 mg/kg PF-2341066 or the vehicle (water, pH 4.0) began. Treatment lasted for 35 days and was given 6 days/week by oral gavage. At the end of the treatment period, the mice were killed, and tumor burden was analyzed by excision of the tumors to determine total tumor weight and the number of metastasis. Tissue for the zymogram was snap-frozen and then stored at -80°C until processed. For the survival study, mice were injected i.p. with SKOV3ip1 cells and treatment with 50 mg/kg PF-2341066 or the vehicle began 8 days after injection. Treatment was continued p.o. 6 days/week, until mice began to show signs of distress (weight loss, ascites, reduced activity) and required euthanization. After 78 days (10 weeks) of survival, all remaining mice were killed, and the presence of tumor was confirmed. Three of the treated mice were censored. In addition, three control mice were not included in the analysis because they never developed tumors. Procedures involving animals were approved by the Institutional Committee on Animal Care, University of Chicago.

Western Blot Analysis

For c-Met and downstream signaling proteins, cells were starved in serum-free medium before being stimulated with HGF/SF (40 ng/ml). Cells were lysed using ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl; pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4 , protease inhibitor cocktail (1:1000; Sigma)). An equal amount (15 μg) of cell extracts was separated by 4% to 20% gradient SDS-PAGE and transferred to nitrocellulose membranes. The transferred samples were incubated with the primary antibody, as indicated in the figure legends, and then incubated with the corresponding secondary horseradish peroxidase-conjugated immunoglobulin G. The proteins were then visualized with enhanced chemiluminescence reagents. Blots were stripped and reprobed for β -actin.

Branching Morphogenesis Assay

The assay was performed as previously described [22]. Briefly, 2000 cancer cells were seeded in 100 μl of a 1:1 mix of growth factor-reduced Matrigel and serum free DMEM, in a 96-well plate. The Matrigel plug was allowed to gel for 30 minutes at 37°C . Then, 100 μl of the medium supplemented with FBS, 40 ng/ml of HGF, and 1 μM PF-2341066 or vehicle was placed on top. The cells were then cultured for 4 days, and pictures were taken using an Axiovert 100 microscope (Zeiss, Göttingen, Germany) at 20 \times . Medium was changed daily.

Proliferation Assay

The proliferation of ovarian cancer cells was measured using the CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR).

The CyQuant GR fluorescence dye shows strong fluorescence enhancement when bound to nucleic acids. SKOV3ip1 or HeyA8 (3×10^3) were seeded in 96-well plates and cultured in the presence of PF-2341066 or an equivalent volume of vehicle. At the indicated time points, cells were washed once with PBS and frozen at -80°C . Cells from each time point were lysed in a buffer containing the fluorescent dye. Fluorescence was measured using a fluorescence spectrophotometer (Synergy HT; Bio-Tek, Winoosky, VT) multiwell fluorescence plate reader at 485 nm excitation and 530 nm emission.

Nuclear Fragmentation to Determine Apoptosis

HeyA8 cells were treated for 48 hours with 10 μM PF-2341066 or vehicle. Cells were rinsed and then fixed with 4% formaldehyde for 15 minutes at room temperature (RT). After fixation, the cells were rinsed twice with Hank's balanced salt solution and then incubated with 2 μM Hoechst 33342 stain (Molecular Probes) for 10 minutes at 37°C . Cells were visualized at $20\times$ using an Axiovert 100 equipped with a digital camera.

Apoptosis and Cell Cycle Analysis

To determine the amount of apoptosis after PF-2341066 treatment, HeyA8 cells were treated for 48 hours with 10 μM PF-2341066 or vehicle. For analysis of cell cycle, HeyA8 cells were serum-starved for 24 hours before treatment with 10 μM PF-2341066 or vehicle for 24 hours. The total cell population was collected, fixed in 70% EtOH, resuspended in propidium iodide staining solution, and stained for 30 minutes in the dark at RT. The samples were then analyzed by flow cytometry for sub- G_1 , G_1 , S, and G_2/M populations using FlowJo software.

In Vitro Adhesion Assay to ECM Components

Ovarian cancer cells were fluorescently labeled with 10 μM CMFDA (green) (Molecular Probes) and then pretreated with 10 μM PF-2341066 for 10 minutes in serum-free media. Cells (5×10^4) were plated in a 96-well plate that had been precoated with collagen type 1 (50 $\mu\text{g}/\text{ml}$), fibronectin (5 $\mu\text{g}/\text{ml}$), or vitronectin (5 $\mu\text{g}/\text{ml}$). After incubating for 30 minutes at 37°C , cells were washed three times (PBS) and fixed with 4% paraformaldehyde for 10 minutes at RT. The number of adhesive cells was quantified by measuring the fluorescent intensity ($\text{Ex} = 590 \text{ nm}$, $\text{Em} = 620 \text{ nm}$) with a fluorescence spectrophotometer (Synergy HT; Bio-Tek).

Gelatin Zymogram

Snap-frozen tumors were pulverized using a mortar and pestle in liquid nitrogen and then lysed in eight-fold volume of RIPA buffer in the absence of a reducing agent [23]. Protein concentrations were determined using Pierce bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL). Equal amounts of protein (30 μg) from three treated and three control mice were combined and resolved on a 10% SDS-polyacrylamide gel containing 0.1% (wt/vol) gelatin as substrate (Bio-Rad, Hercules, CA). The gel was then rinsed in Triton-X buffer (2.5% Triton X-100, 50 mM Tris-HCl (pH 7.5), 0.05% NaN_3) for 2 hours at RT with gentle shaking to rinse out the SDS and allow the protein to renature. Then the gel was incubated overnight at 37°C in incubation buffer (0.15 M NaCl, 50 mM Tris-HCl, 10 mM CaCl_2 , 0.05% NaN_3) that activates the collagenase. After the overnight incubation, the gel was stained with 0.25%

Coomassie blue (45.5% MeOH, 1.1% AcCOOH) for 45 minutes at RT. After staining, the gel was destained with a solution containing 10% AcCOOH and 25% MeOH for 3 hours at RT with three washes on a shaker until contrast is satisfactory. Conditioned medium from HT-1080 cells was used as a positive control for the full and active forms of matrix metalloproteinases 9 and 2 (MMP-9 and MMP-2). Proteolysis was detected as a white zone in a dark field.

Immunohistochemistry

Tumors extracted from mouse omentum were formalin-fixed, paraffin-embedded, sectioned, and mounted on slides. To retrieve antigens, slides were boiled in 0.01 M sodium citrate pH 5.0 for 20 minutes. All slides were blocked in avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA). Slides were stained with hematoxylin and eosin (H&E) or incubated with primary antibodies against Ki-67 (1:300; LABVISION, Fremont, CA) and CD31 (1:200; Santa Cruz Biotechnology) for 1 hour. After three washes in Tris-buffered saline (TBS), the slides were incubated with the species-appropriate biotinylated secondary antibody (1:500). Again, the slides were washed with TBS then incubated with peroxidase-linked avidin using the Vectastain ABC kit (Vector Laboratories) for 30 minutes. The slides were rinsed in TBS and stained with 3-3'-diaminobenzidine chromogen. Negative controls were prepared by omitting the primary antibody.

Statistical Analysis

Overall survival estimates were computed using the Kaplan-Meier method, and comparisons between groups were analyzed using the log-rank test. Significant changes were determined by two-sided, unpaired t test.

Results

PF-2341066 Inhibits In Vivo Tumor Growth

As a first step toward determining the potential therapeutic efficiency of PF-2341066, we used an ovarian cancer xenograft model in which SKOV3ip1 ovarian cancer cells are injected intraperitoneally (i.p.) into the abdominal cavity of nude mice [21]. After tumors were established for 8 days, mice underwent oral treatment with either PF-2341066 or the vehicle for 4 weeks. In mice treated with the inhibitor, the number of metastases (Figure 1A) was reduced by 63% ($P < .0001$) and the overall tumor weight (Figure 1B) was reduced by 55% ($P < .0005$) compared with the control mice. There was no significant difference in the ascites volume between treated and control groups (data not shown). Microscopic evaluation of H&E-stained tumors revealed large areas of necrosis in tumors from treated mice (Figure 1C). The effect of the inhibitor on proliferation (Ki-67) and angiogenesis (CD31) was evaluated by immunohistochemistry. Tumors from treated mice had a marked reduction in Ki-67 staining (Figure 1C) compared with the control tumors; however, there was no significant change in CD31 staining (data not shown). Because treatment with PF-2341066 significantly reduced tumor burden, we sought to determine whether treatment could also increase survival time. Mice with established i.p. tumors were treated either with PF-2341066 or vehicle for 10 weeks. Treatment with the c-Met inhibitor increased the median survival time of mice to 62 days compared with the mice receiving vehicle, which became distressed after 45 days (Figure 2). Log-rank analysis (Kaplan-Meier) showed that the survival benefit from treatment with PF-2341066 was significant ($P = .0003$).

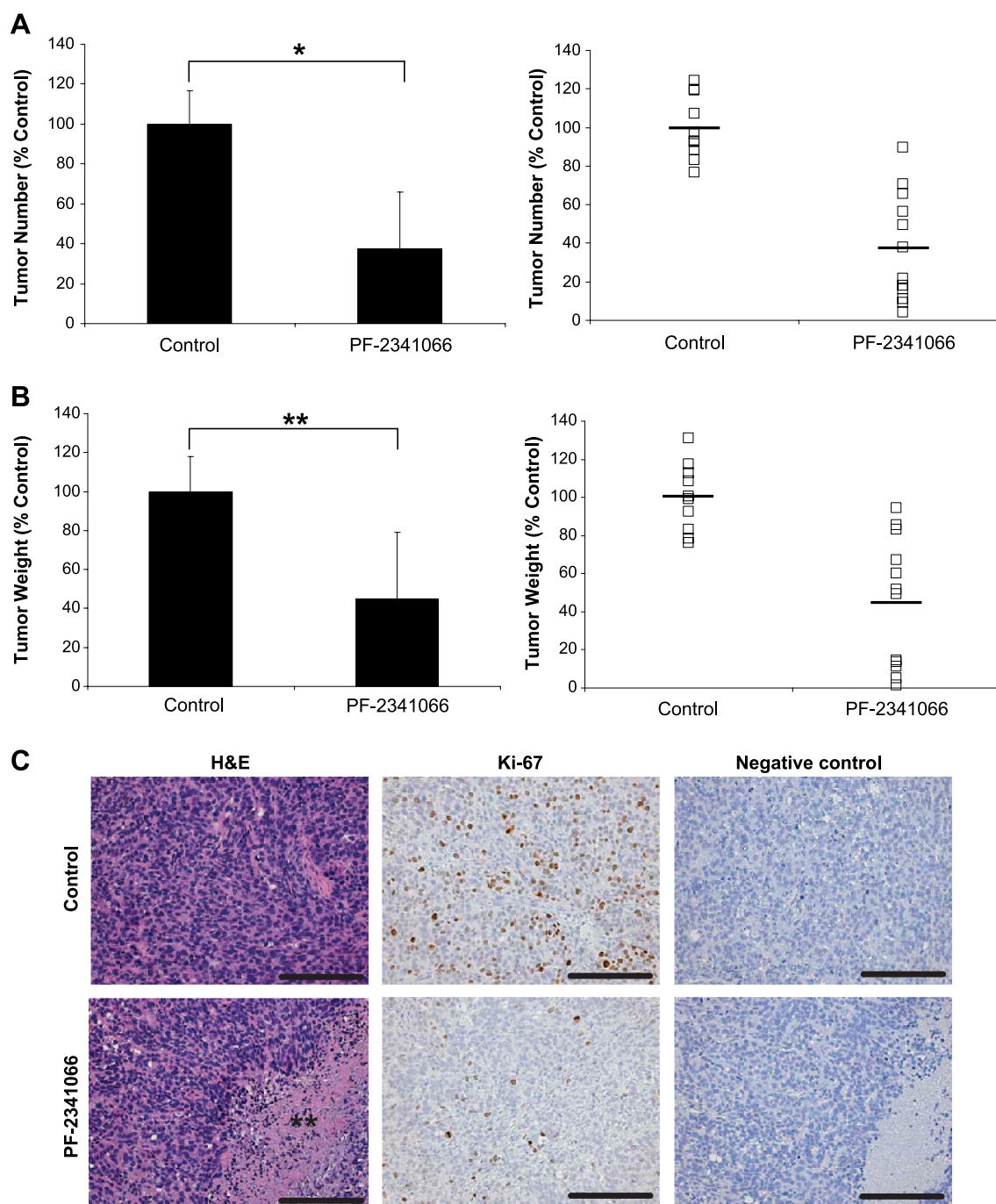


Figure 1. Treatment with PF-2341066 reduces tumor burden in an ovarian cancer xenograft model. Female athymic nude mice bearing SKOV3ip1 i.p. tumors (8 days) were given PF-2341066 at 50 mg/kg or vehicle 6 days/week by oral gavage for 5 weeks. At the end of the experiment, tumors were counted to determine tumor number (A) and weighed (B) to assess tumor burden. Each square indicates individual mouse (right). (A, B) Treated $n = 12$, control $n = 10$. $*P < .0001$, $**P < .0005$. (C) Immunohistochemistry of tumors from control- and PF-2341066-treated mice. H&E, Ki-67, and negative control are shown. Representative pictures are shown for the treatment groups. Original magnification, $\times 200$. Scale bar, 200 μm . **Area of necrosis in PF-2341066-treated tumors.

Effects of PF-2341066 on c-Met Phosphorylation and Downstream Signaling in Ovarian Cancer Cell Lines

PF-2341066 had been previously shown to be a potent inhibitor of c-Met and NPM-ALK kinase [18,19]. A thorough review of the literature and available databases revealed that ALK or its oncogenic fusion variants have never been shown to be expressed or active in ovarian cancer tumors. We confirmed this by Western blot analysis

showing that ovarian cancer cell lines do not express ALK or ALK-fusion proteins compared with lysates from an anaplastic large cell lymphoma (ALCL) cell line, SUDHL-1 (Figure 3A). We therefore focused our studies of PF-2341066 in ovarian cancer on its anti-c-Met effect. Stimulation with HGF leads to activation of c-Met and subsequent activation of MAPK, PI3K, and signal transducers and activators of transcription signaling pathways that mediate the biologic

effects of c-Met activation [4,5]. Therefore, we sought to understand if PF-2341066 inhibits the HGF/c-Met signaling pathway. Three ovarian cancer cell lines (CaOV3, SKOV3ip1, and HeyA8) were pre-treated with the indicated doses of PF-2341066 before stimulation with HGF. All three cell lines expressed c-Met, the highest expression occurring in CaOV3 cells (Figure 3B). Pretreatment with 50 nM PF-2341066 was sufficient to block HGF induced c-Met phosphorylation by more than 50% in all three cell lines (Figure 3B). Complete inhibition of c-Met was achieved in both CaOV3 (Figure 3B, *left panel*) and SKOV3ip1 (Figure 3B, *middle panel*) cells with a 1- μ M dose as shown by an absent phosphorylation of tyrosine residues 1230/1234/1235, which are localized in the tyrosine kinase domain. PF-2341066 blocked the phosphorylation of Akt and Erk1/Erk2, suggesting inhibition of the c-Met-dependent pathways, PI3K and Erk-MAPK (Figure 3B).

The HeyA8 ovarian cancer cells [24] behaved differently than the SKOV3ip1 and CaOV3 cells: phosphorylation of c-Met at residues in the kinase domain (1230/34/35) could only be partially inhibited by the highest dose of PF-2341066, and the inhibitor had no effect on the phosphorylation of Erk1/Erk2. However, the phosphorylation of Akt was clearly inhibited by PF-2341066 (Ser 473) (Figure 3B, *right panel*). Further analysis of HeyA8 signaling revealed that the inhibitor reduced c-Met phosphorylation in unstimulated cells at the Gab-1 (Grb2-associated binding protein-1) binding site, tyrosine 1349 (Figure 3C). Activation of the known downstream signal transducers, Gab-1 and Src, were both inhibited by treatment with PF-2341066 (Figure 3C). In addition, phosphorylation of FAK (focal adhesion kinase) at the major Src binding site, 861, was reduced by the inhibitor.

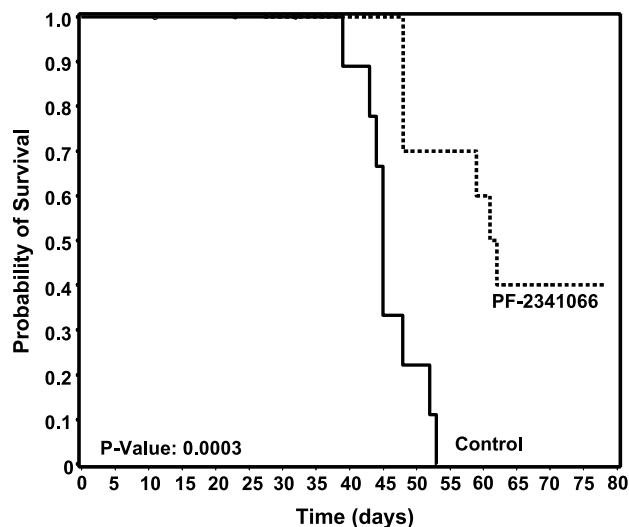


Figure 2. Treatment with PF-2341066 increases survival. Female athymic nude mice were injected i.p. with SKOV3ip1 cells and, after 8 days, were given PF-2341066 at 50 mg/kg or vehicle 6 days/week by oral gavage. Treatment continued until mice began to show signs of distress and then mice were killed and the presence of tumor was confirmed. All living mice were killed after 10 weeks of survival, and the presence of tumor was confirmed. Survival curves were calculated using Kaplan-Meier. Treatment with PF-2341066 significantly improved the overall survival of cancer-bearing mice ($P = .0003$). Control $n = 9$, treated (PF) $n = 10$.

The biologic effect of c-Met activation results in a morphogenic response known as “invasive growth.” This can be visualized using the branching morphogenesis assay that measures the ability of cells to grow and invade in a three-dimensional matrix [22]. To determine the effect of PF-2341066 treatment on branching morphogenesis, HeyA8 and SKOV3ip1 cells were seeded within a three-dimensional matrix. In the presence of HGF, the cells formed multicellular-branched structures (Figure 3D). Addition of the inhibitor, PF-2341066, was sufficient to inhibit branching morphogenesis in both HeyA8 and SKOV3ip1 cells (Figure 3D).

PF-2341066 Inhibits Adhesion and Invasion of Ovarian Cancer Cells

One of the key steps in ovarian cancer metastasis is the attachment of cancer cells to peritoneal surfaces within the abdominal cavity, most commonly the peritoneum and omentum [25]. We had previously shown that silencing c-Met with siRNA reduced the adhesion of ovarian cancer cells to peritoneum and different ECMs [6]. Therefore, we analyzed the effect of c-Met inhibition by PF-2341066 on ovarian cancer cell adhesion to several ECMs previously shown to be involved in early ovarian cancer metastasis [26]. Fluorescently labeled ovarian cancer cells were pretreated with PF-2341066 or vehicle (mock) and then allowed to adhere to plastic, collagen type I, fibronectin, or vitronectin. The number of adherent cells was quantified by measuring fluorescence, which represents the number of attached cells (Figure 4A). Treatment with the c-Met inhibitor significantly reduced the adhesion of both SKOV3ip1 and HeyA8 (Figure 4A) to all the ECMs, with the most significant inhibition shown in adhesion to fibronectin. MMPs have been shown to play an important role in tumor progression *in vitro* and in animal models. Beyond their ability to facilitate migration and invasion through degradation of ECMs, they are also known to release mitogenic growth factors from cell surfaces and from ECM reservoirs and to regulate tumor-associated angiogenesis [27]. The expression of MMP-2 and MMP-9, in particular, has been shown to correlate with a greater metastatic potential in ovarian tumors [28]. Therefore, to determine whether the activity of key proteases is reduced by treatment with PF-2341066, we analyzed MMP-2/9 gelatinolytic activity in SKOV3ip1 tumor xenografts from Figure 1 using zymography. Tumors from vehicle-treated mice were found to secrete an 83-kDa gelatinase indistinguishable in size from the active form of MMP-9 (Figure 4B) and a 66-kDa gelatinase consistent with the active form of MMP-2. Tumors from mice treated with PF-2341066 displayed reduced activity of both enzymes when compared with the tumors from control mice (Figure 4B).

Treatment with PF-2341066 Reduces Ovarian Cancer Cell Growth and Induces Apoptosis

Key mediators of tumor progression are proliferation and resistance to apoptosis. A fluorescence-based proliferation assay was used to determine whether treatment with PF-2341066 inhibits ovarian cancer cell growth. The two ovarian cancer cell lines, SKOV3ip1 and HeyA8, were treated with either PF-2341066 or vehicle (mock). A modest but significant reduction in proliferation was observed with a 1- μ M dose in both SKOV3ip1 and HeyA8 to day 3 (Figure 4C). Cell cycle analysis showed a potent G₂/M arrest in HeyA8 cells treated with the inhibitor (Figure 4D). The next step was to determine whether treatment with PF-2341066 could also induce cell death. HeyA8 cells were incubated with PF-2341066 or vehicle for 48 hours, and nonadherent cells were collected. Staining of the treated cells with

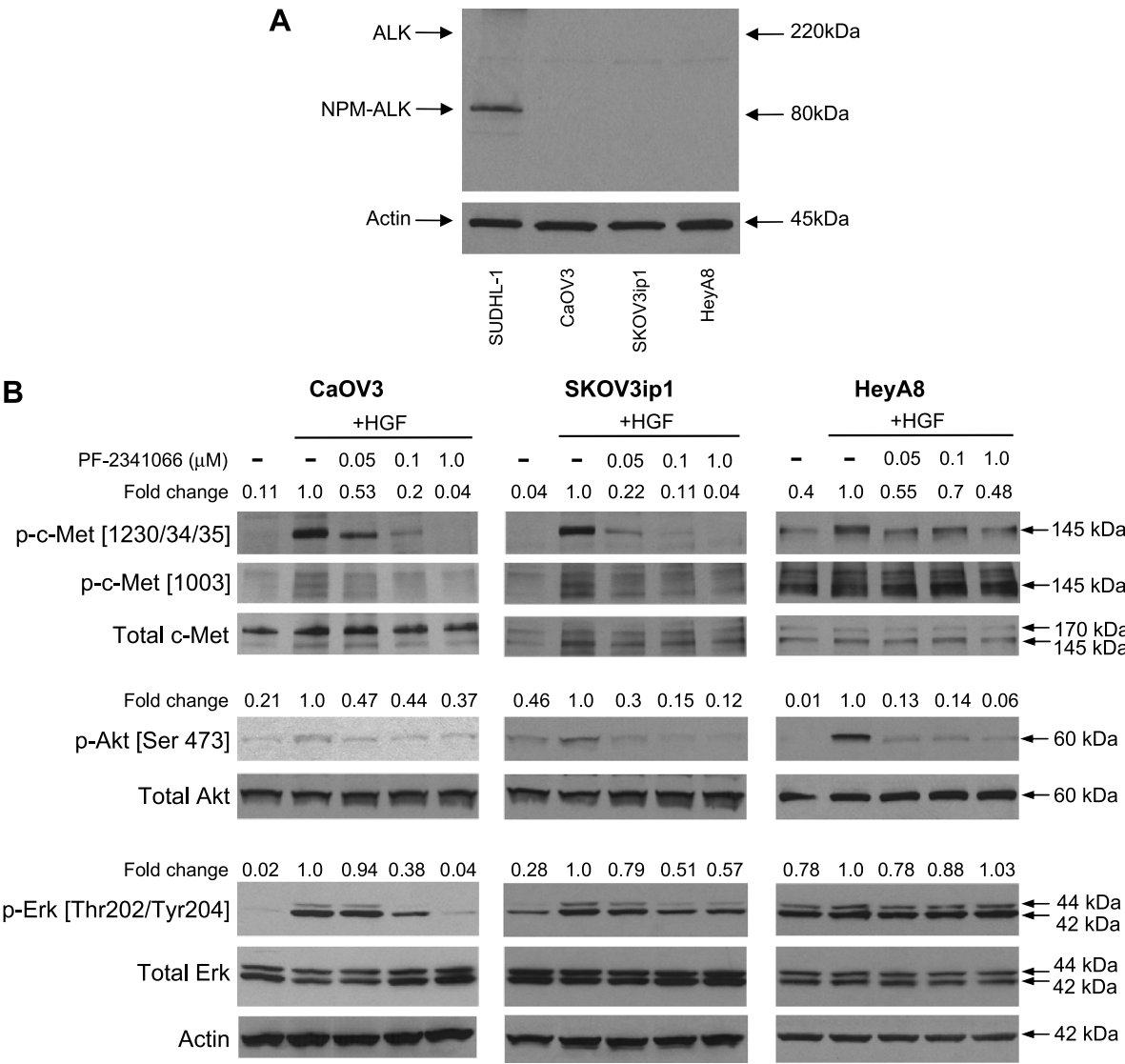


Figure 3. PF-2341066 inhibits c-Met and downstream signaling *in vitro*. (A) Western blot showing expression of ALK in CaOV3, SKOV3ip1, and HeyA8 cell lines using an antibody that recognizes both wild type and fusion ALK proteins. SUDHL-1 is a positive control expressing NPM-ALK and does not express full-length ALK. (B) Western blots showing expression of c-Met and downstream signaling proteins. The ovarian cancer cell lines, CaOV3, SKOV3ip1, and HeyA8, were serum-starved for 24 hours and then pretreated with the indicated concentrations of PF-2341066 for 1 hour before stimulation with 40 ng/ml HGF for 10 minutes. (C) Western blots of phospho-proteins; c-Met, Gab-1, Src, and FAK. HeyA8 cells were serum-starved for 24 hours before treating with 1 μM PF-2341066 or vehicle. (D) Branching morphogenesis assay. SKOV3ip1 and HeyA8 cells were mixed with Matrigel, plated, and cultured for 4 days in complete medium supplemented with 40 ng/ml HGF with or without 1 μM PF-2341066. Original magnification, ×20.

Hoechst 33342 showed nuclear condensation and DNA fragmentation (Figure 4E, left), hallmarks of apoptotic cell death. In addition, flow cytometry analysis of cells stained with propidium iodide showed a near two-fold increase in apoptotic cell population in treated cells (Figure 4E, right).

Discussion

The therapeutic efficacy of c-Met inhibition has been studied in a number of preclinical models. However, many of the inhibitors studied are not suitable for patient treatment because of poor pharmaceutical properties [12]. PF-2341066 is a highly specific, orally available, c-Met inhibitor that is presently in phase 1/2 clinical studies and could be considered for clinical use in ovarian cancer. Herein we

show that treatment with the inhibitor abrogates HGF-stimulated c-Met activation and downstream signaling as well as branching morphogenesis. Furthermore, PF-2341066 reduced proliferation, adhesion, and protease expression and induced apoptosis. To the best of our knowledge, this is the first study to show that a small-molecule inhibitor targeting c-Met, PF-2341066, is effective in reducing tumor burden and increasing survival in a preclinical model of ovarian cancer. The invasive growth program initiated by HGF activation of c-Met has been shown to be dependent on the coordinated activation of both the Ras and PI3K pathways [29]. Although c-Met phosphorylation could be similarly inhibited by PF-2341066 in all cell lines tested, differential behavior of downstream signaling pathways was observed. Notably, phosphorylation of Erk1/2 was not affected by HGF stimulation or c-Met inhibition in HeyA8 cells compared with

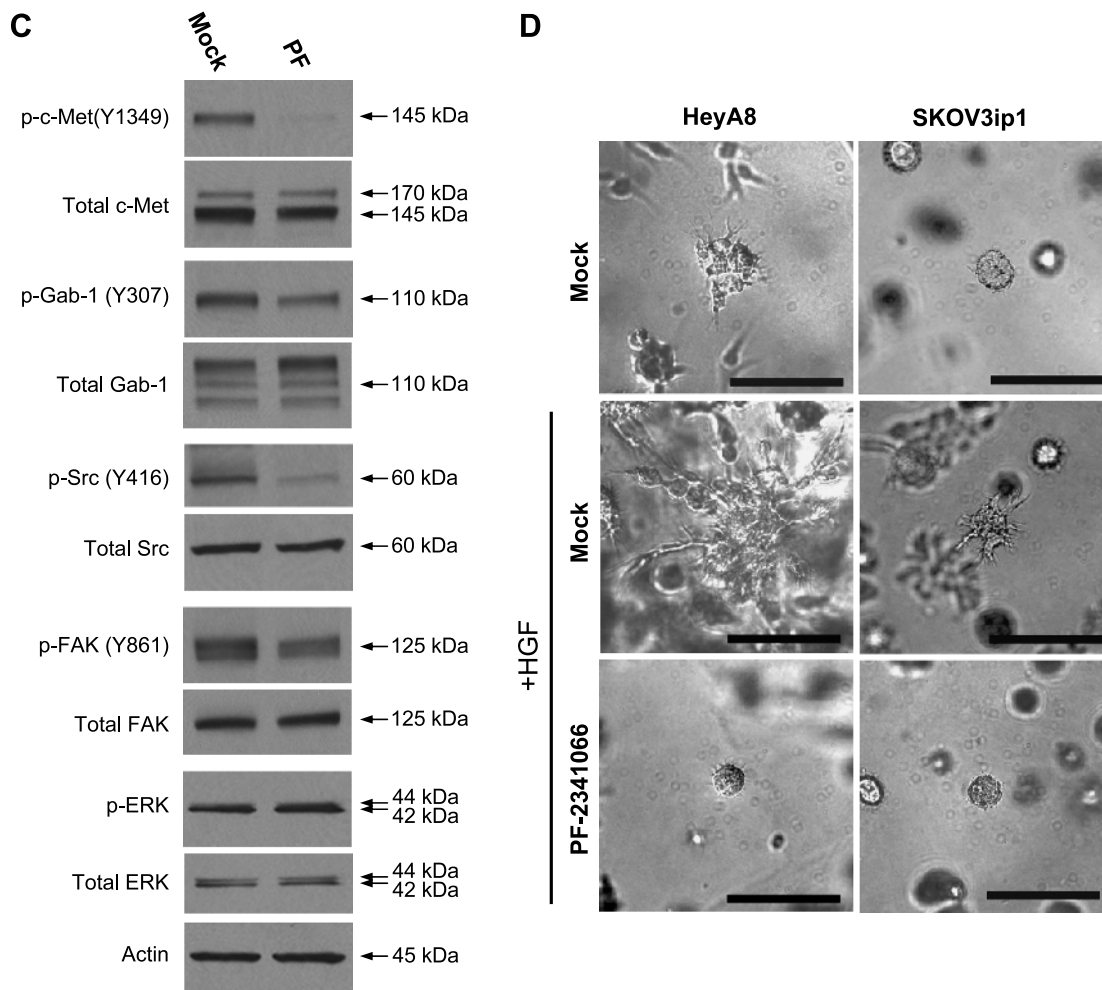


Figure 3. (continued).

SKOV3ip1 cells. A possible explanation for this effect might be that the HeyA8 cell line contains a *BRAF* mutation that results in constitutive activation of the Ras/Raf/Erk pathway [30]. Further analysis of downstream signaling in HeyA8 cells revealed that activation of the signal transducers, Gab-1 and Src, was both reduced with PF-2341066 treatment. Phosphorylation of c-Met at site 1349 has been shown to be critical for Gab-1 and Src binding and, consequently, necessary for c-Met's biologic function [31–33]. Therefore, these findings suggest that PF-2341066 is sufficient to inhibit c-Met dependent growth and morphogenesis.

The *in vivo* studies showed that, tumor weight was inhibited by 55% and the number of metastases was inhibited by approximately 63%. These data are consistent with the report from Saga et al. [34], which showed an approximate 70% inhibition of metastasis with an ovarian cancer cell line stably transfected with the HGF competitive molecule, NK4. In addition, we previously reported that silencing of c-Met through siRNA treatment in an *in vivo* model of ovarian cancer resulted in an 85% inhibition of tumor nodules, tumor weight, and ascites volume [6]. Owing to the specific nature of siRNA as well as the use of the identical cell line in both studies, the comparable effects observed with PF-2341066 treatment can likely be attributed to the specific inhibition of c-Met. Other small-molecule inhibitors of c-Met have been shown to be effective in preclinical models of lung cancer [35] and in models of metastasis [16], which supports

the therapeutic relevance of targeting c-Met. Zou et al. [19] showed that treatment with PF-2341066 was sufficient to inhibit 100% of tumor growth in a subcutaneous mouse model. As a strategy to bypass the problem of the incompatibility of mouse HGF with human c-Met, the authors used models with constitutively active c-Met. For example, the GTL-16 cell line is a human gastric carcinoma that expresses high levels of constitutively active c-Met and the U87G human glioblastoma cell line expresses both HGF and c-Met, comprising an autocrine loop. The dependency of these cell lines on c-Met makes them useful for the study of c-Met inhibition by PF-2341066. However, it is likely that these cell line models are representative of only a subset of very c-Met-dependent cancers.

In our model, c-Met is not constitutively active, nor does it express HGF (data not shown). Therefore, this model probably represents a wider range of ovarian cancers (and other tumors) in which HGF–c-Met signaling is only one of many activators of oncogenic signaling pathways contributing to tumorigenesis. That the inhibitor only reduced tumor growth and metastasis by around 60% indicates that oncogenic pathways other than c-Met are important in the growth of ovarian cancer cells. Therefore, combining a c-Met inhibitor with other targeted treatments or chemotherapy might be an effective next step in ovarian cancer therapeutics. Obvious candidates for combined therapy with PF-2341066 are paclitaxel and carboplatin, the drugs commonly used for ovarian cancer treatment.

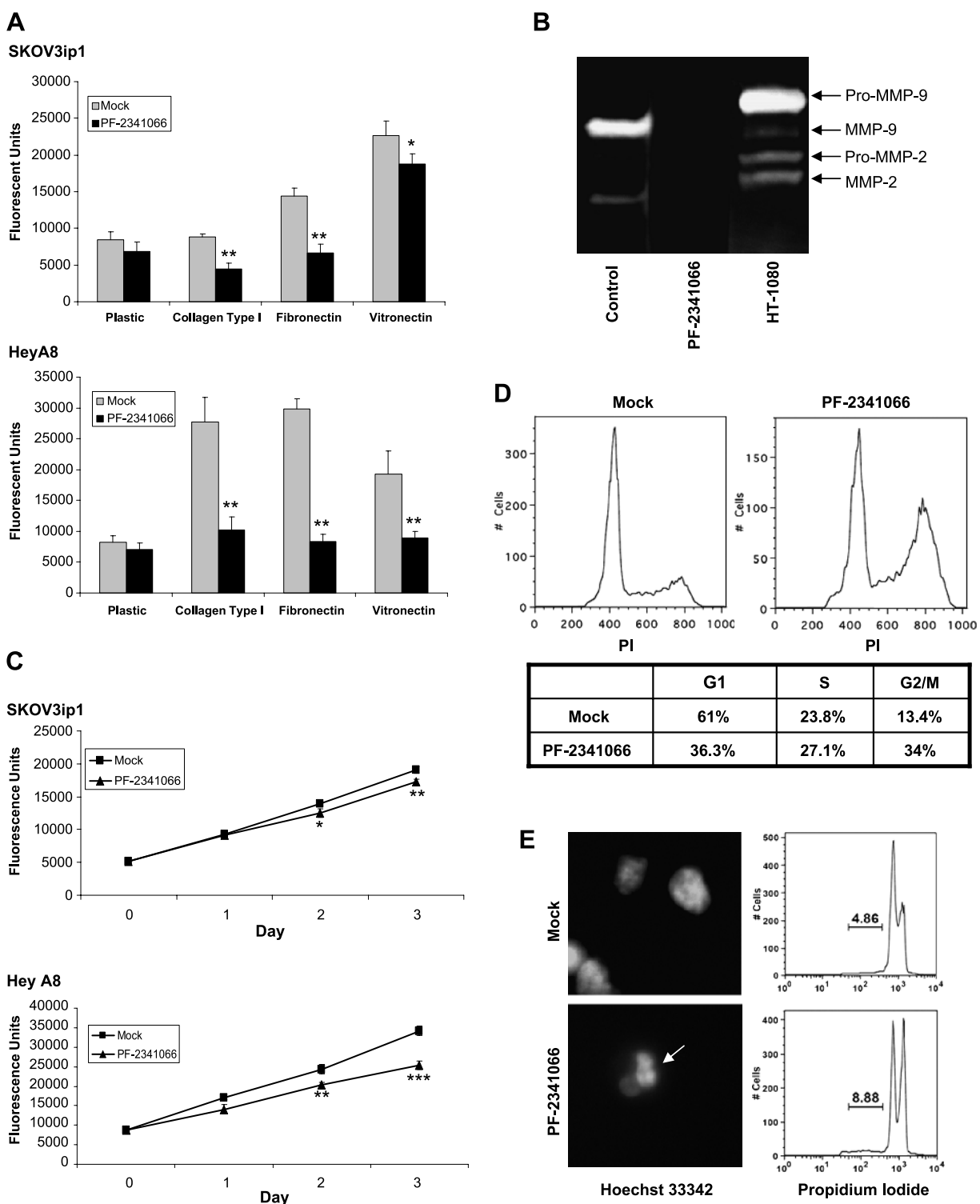


Figure 4. PF-2341066 inhibits proliferation, adhesion, and invasion and induces cell death in ovarian cancer cell lines. (A) Adhesion assay. *In vitro* adhesion assay to ECM components. SKOV3ip1 and HeyA8 cells were fluorescently labeled with CMFDA and then pretreated with 10 μ M PF-2341066 for 10 minutes. After incubation for 30 minutes with the indicated ECMs, the number of adhesive cells was determined using a fluorescence reader. * $P < .01$, ** $P < .0001$. (B) Gelatin zymogram. Tumors from mice treated with either PF-2341066 or vehicle (control) were lysed in RIPA buffer and resolved on a 10% SDS-PAGE gel containing gelatin. HT-1080 conditioned medium served as a positive control. (C) Proliferation assay. SKOV3ip1 and HeyA8 cells were plated in complete medium with either 1 μ M PF-2341066 or vehicle and assayed at the indicated time points. * $P < .005$, ** $P < .0001$, *** $P < .00001$. (D) Cell cycle analysis. HeyA8 cells were serum-starved for 24 hours before treating with PF-2341066 for 24 hours. Cells were then stained with propidium iodide and analyzed using a flow cytometer. The percentage of cells in each phase of the cell cycle is shown. (E) Nuclear fragmentation (left). SKOV3ip1 cells were treated for 48 hours with 10 μ M PF-2341066 or vehicle. Nonadherent cells were collected by centrifugation and stained with 2 μ M Hoechst 33342 to visualize cell death. Cells were visualized at a magnification of $\times 20$. Quantification of apoptosis by flow cytometry (right). Cells were treated for 48 hours with 10 μ M PF-2341066 or vehicle, stained with propidium iodide, and analyzed using a flow cytometer. Percent of sub-G₁ is indicated.

Whereas PF-2341066 has also been shown to inhibit oncogenic fusion and mutant variants of ALK [18], these proteins have most frequently been reported to be expressed in ALK-positive cases of ALCL, inflammatory myofibroblastic tumors, and, more recently, a small subset of solid tumors [36–38]. We did not find evidence of expression in ovarian cancer cell lines, and an extensive search of the literature revealed no evidence of ALK expression or activity in adult ovarian tissues. ALK is shown to have only low expression in adult mammalian tissues, and whereas ALK fusion proteins are shown to have oncogenic properties in ALCL, no pathologic function of wild type ALK has been described [39]. Despite the noted importance of ALK in neuronal development (reviewed in Allouche [40]), the physiological role of ALK in the adult has not yet been fully elucidated because ALK-null mice are shown to exhibit a normal phenotype [37]. These findings suggest that there may be few off-target adverse effects in adult patients treated with PF-2341066.

In summary, this study shows that PF-2341066 effectively reduces tumor burden, thereby increasing median survival time, in an *in vivo* model of ovarian cancer metastasis. The data presented here indicate that PF-2341066 should be studied further as a potential therapeutic in ovarian cancer. Last but not least, the oral availability of PF-2341066 offers patients a mode of administration far less inconvenient and uncomfortable than intravenous treatments.

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